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(54) Title: *IN VITRO* PROPAGATION OF EMBRYONIC STEM CELLS

(57) Abstract

The present invention relates generally to the use of leukaemia inhibitory factor (LIF) in the maintenance and derivation of embryonic stem (ES) cells in culture. The ES cells are maintained and/or derived from animal embryos by culturing said cells or embryos in a culture medium containing an effective amount of LIF for a time and under conditions sufficient to maintain and/or derive said ES cells. The ES cells may be passaged in LIF and used to make chimaeric animals.

IN VITRO PROPAGATION OF EMBRYONIC STEM CELLS

This invention relates to the use of a previously discovered and characterised molecule, leukaemia inhibitory factor (LIF), in the isolation and propagation of embryonic stem cells in vitro.

Embryonic stem (ES) cells, the pluripotent outgrowths of blastocysts, can be cultured and manipulated in vitro and then returned to the embryonic environment to contribute normally to all tissues including the germline (for review see Robertson, E.J. (1986) Trends in Genetics 2:9-13). Not only can ES cells propagated in vitro contribute efficiently to the formation of chimaeras, including germline chimaeras, but in addition, these cells can be manipulated in vitro without losing their capacity to generate germ-line chimaeras (Robertson, E.J. et.al. (1986) Nature 323:445-447).

ES cells thus provide a route for the generation of transgenic animals such as transgenic mice, a route which has a number of important advantages compared with more conventional techniques, such as zygote injection and

In work leading to the present invention, it has been found that LIF has the capacity to substitute for, or be added to, feeder layers (or conditioned medium) in supporting the maintenance of pluripotential ES cells in vitro.

LIF is a protein that has previously been purified, cloned and produced in large quantities in purified recombinant form from both Escherichia coli and yeast cells. (International Patent Application No. PCT/AU88/00093, filed March 31, 1988.) LIF has been defined as a factor, the properties of which include:

1. it has the ability to suppress the proliferation of myeloid leukaemic cells such as Ml cells, with associated differentiation of the leukaemic cells; and

2. it will compete with a molecule having the defined sequence of murine LIF or human LIF (defined in International Patent Application No. PCT/AU88/00093) for binding to specific cellular receptors on Ml cells or murine or human macrophages. In addition to the biological properties previously disclosed for murine and human LIF, LIF has now been found to have the following properties:

(a) it allows the derivation and maintenance in the absence of feeder cells of the pluripotential phenotype in vitro of ES cells.

(b) it allows the aforementioned ES cells, after passage in vitro in the presence of LIF, to contribute to somatic and germline cell tissues of chimaeric animals such as mice when re-introduced into the embryonic environment;

(c) it demonstrates selective binding to high affinity receptors on murine ES (EKcs-1 (previously known as CS1) and D3) and EC (PCC3-3A and F9) cells; and

or conditioned medium in maintaining ES cells in vitro. For the purposes of the present description recombinant LIF is produced in E. coli and yeast using the methods described in International Patent Application No. PCT/AU88/00093, however, it is within the scope of the present invention to include recombinant LIF produced in other hosts including mammalian and insect cells and to synthetic LIF.

In another aspect, the present invention extends to ES cells derived from animal embryos by passage in a culture medium containing LIF, to such ES cells having additional genetic material inserted therein, and to chimaeric animals such as chimaeric mice or transgenic progeny of said animals generated by known techniques using ES cells which have been maintained in vitro in a LIF-containing culture medium.

Thus, the invention extends to the generation and maintenance of ES cells from humans, mice, birds (eg. chickens), sheep, pigs, cattle, goats and fish and to the generation of transgenic chimaeric animals and their transgenic progeny using the ES cells isolated from animal species such as mice, birds (eg. chickens), sheep, pigs, cattle, goats and fish. This invention also includes the use of LIF in culture media to modulate the survival and growth of human and other animal species such as cattle germ cells and embryonic cells, for example, for use in in vitro fertilisation and other procedures.

The present invention may also be described by reference to the following figures:

Figure 1 is a graphical representation showing the effect on ES cells of different concentrations of LIF.

Figure 2 is a pictorial representation showing ES cell morphology in the presence and absence of LIF.

modified Eagle's medium with supplements such as 5% - 30% (v/v) foetal calf serum and where necessary 0.01 to 1.0 mM ß-mercaptoethanol but preferably about 0.1 mm ß-mercaptoethanol. The culture medium may or may not contain feeder cells and LIF may be used to substitute for, or add to, said feeder cells. When required, LIF, or more particularly synthetic or recombinant LIF, is added to the medium at a concentration of about 100 - 1,000,000 units/ml and preferably about 100 - 100,000 units/ml and even more preferably 500 - 10,000 units/ml where 50 units are defined as the amount of LIF which in one millilitre induces a 50% reduction in clone formation by murine M1 myeloid cells. By "recombinant LIF" is meant the LIF prepared by genetic engineering means such as, for example, according to International Patent Application No. PCT/AU88/00093 where a number of hosts such as bacteria (eg. E. coli) or yeast cells may be employed. In accordance with the present invention, the effective derivation time is from 1 day to 20 weeks and particularly from 1 to 8 weeks.

Another aspect of the present invention contemplates a process for maintaining animal ES cells in vitro while retaining their pluripotential phenotype which process comprises culturing said cells in a culture medium containing an effective amount of LIF under conditions sufficient to maintain said cells. The ES cells in accordance with this aspect of the invention include cells derived from humans, mice, birds (eg. chickens), sheep, pigs, cattle, goats and fish. As with the isolation of ES cells from animal embryos, the LIF used in the aformentioned process is preferably recombinant LIF. The culture medium may or may not contain feeder cells.

The present invention also relates to chimaeric animals generated by known techniques using the ES cells contemplated herein. These ES cells may be isolated from animal embryos and/or maintained in vitro according to the subject invention. Furthermore, genetically manipulated ES cells may be passaged in LIF and used to make chimaeric animals. For example, genetically manipulated ES cells containing a retrovirus vector (N-TK527; derived from pXT1; C.A. Boulter and E.F. Wagner, (1987) Nucl. Acids Res. 15:7194) encoding genes for neomycin resistance and c-src^{527} were propogated in the presence of LIF but in the absence of feeder cells for over 20 passages. These cells still retained the ability to differentiate as judged by the formation of normal chimaeras following introduction of these cells into preimplantation embryos by blastocyst injection.

Further details of the use of LIF in accordance with the present invention will be apparent from the following Examples.

EXAMPLE 1

This example sets out the steps used to maintain ES cells in vitro in LIF, and to generate chimaeric mice using ES cells so passaged.

Step 1: Propagation in vitro:

The ES cells used were the D3 (Doetschman, T.C. et.al. (1985) J.Embryol.Exp.Morphol. 87:27-45) the EKcs-1 (previously known as CS1) (Wagner, E.F. et.al. (1985) Cold Spring Harbor Symp.Quant.Biol. 50:691-700) and the HD5 (C. Stewart, unpublished) ES cell lines isolated from 129 SV He blastocysts and the CBL63 (R.Kemler, unpublished) ES cells isolated from C57BL/6J blastocysts. Prior to

phenotype by transfer to media containing 1,000 units ml^{-1} M-LIF (A), or to normal culture media (B). After seven days, the colonies were stained with Giemsa. Compact stem-cell colonies could be distinguished from diffuse differentiated colonies. D3 cells maintained in H-LIF for 15 passages were assayed for the ability to differentiate by transfer into media containing 1,000 units ml^{-1} M-LIF (C) or normal culture media (D). Immunofluorescence of the cells in the two D3 colony types was carried out using the ECMA-7 monoclonal antibody which recognizes a stem cell-specific cell-surface antigen. Cell-surface-specific immunofluorescence was detected on over 90% of the cells maintained in media containing 1,000 units ml^{-1} recombinant LIF (E) but on less than 1% of the cells maintained in normal culture media (F). The field of view shown in (F) contains 21 cells.

Figures 1 and 2 indicate that over 90% of the ES cells maintained in 1000-5000 units/ml rY-HLIF or rE-MLIF retained their stem cell phenotype. In contrast, ES cells maintained in normal culture medium differentiated over a period of 3-6 days. The different concentrations of rY-HLIF or rE-MLIF used did not result in any noticeable change in cell number after 6 days in culture, indicating that there is no selection for a specific subpopulation able to grow in LIF. Similar results have been obtained using yeast-derived rMLIF also disclosed in International Patent Application No. PCT/AU88/00093. The data in Figure I indicate that human LIF acts on mouse ES cells, as previously described for the action of human LIF on M1 myeloid leukaemic cells (Gough,N.M. et.al. (1988) Proc.Natl.Acad.Sci.USA 85: 2623-2627). The data in Figure I also indicate that the action of LIF on ES cells is independent of glycosylation, as previously described for the action of LIF on M1 myeloid leukaemic cells.

expanded for further analysis. The second method for isolation of ES cell lines used the immunosurgery technique (described in Martin, G. R. (1981) Proc. Natl. Acad. Sci. USA 78:7634-7638) where the trophectoderm cells are destroyed using anti-mouse antibodies prior to explanting the inner cell mass. The efficiency of ES cell lines isolation is shown in Table 1.

Step 3: Generation of Chimaeric Mice:

All the ES cell lines cultured in the absence of feeder cells but in the presence of LIF (referred to in step 1) or directly isolated with the aid of culture medium containing LIF (referred to in step 2) retained the ability to differentiate into multiple cell types following the removal of LIF indicating that these cells have retained their pluripotential phenotype. To confirm their developmental potential, D3 ES cells maintained in LIF for 7-22 passages and MBL-1 ES cells maintained in LIF for 14-17 passages were reintroduced into the embryonic environment by blastocyst injection (as described in Williams et al., (1988) Cell 52:121-131). Blastocysts were isolated from the outbred ICR mouse strain or inbred C57BL/6J mice. The expanded blastocysts were maintained in oil-drop cultures at 4°C for 10 min prior to culture. The ES cells were prepared by picking individual colonies, which were then incubated in phosphate-buffered saline, 0.5 mM EGTA for 5 min; a single cell suspension was prepared by incubation in a trypsin-EDTA solution containing 1% (v/v) chick serum for a further 5 min at 4°C. Five to twenty ES cells (in Dulbecco's modified Eagle's Medium with 10% (v/v) foetal calf serum and 3,000 units/ml DNAase 1 buffered in 20 mM HEPES [pH 8]) were injected into each blastocyst. Blastocysts were transferred into pseudopregnant recipients and allowed to

TABLE 1: Isolation of 129 Sv He ES cell lines in media containing rE-HLIF

Methodology	Blastocyst	ICM outgrowing	Number of ES cell lines derived
Explanted	9	9	4
Immunosurgery	11	3	0
Immunosurgery	7	5	2

Murine blastocysts were isolated from 129 Sv He mice at day 4 of development (day 1 = day of plug) into either Dulbecco's or Glasgows modified Eagle's medium with 15% (v/v) foetal calf serum, 0.1mM ß-mercaptoethanol and 1000 units/ml of purified rE-HLIF. The blastocysts were then explanted into the same media and left to attach to the culture dish and the inner cell mass picked dissociated in phosphate-buffered saline, 0.5 mM EGTA for 5 min; a single cell suspension was prepared by incubation in a trypsin-EDTA solution containing 1% (v/v) chick serum and the cells replated in the cell culture medium described above. The characteristic ES cell colonies appeared within 1 - 3 weeks.

Other blastocysts were treated by immunosurgery (as described in Martin, G. R. (1981) Proc. Natl. Acad. Sci. USA 78:7634-7638). The blastocysts were allowed to hatch from the zona pelucida, and then treated with anti-mouse antibodies and destroyed by the addition of complement. The exposed inner cell mass was then left to

The following relates to Tables 2, 3 and 4:

D3 and MB1-1 ES cells are derived from 129 Sv He mice (inbred, agouti, homozygous for the glucose phosphate isomerase 1^a allele). The D3 ES cells were originally cultured on primary embryo fibroblasts for 10 passages and then transferred to 1,000-5,000 units/ml recombinant LIF for 7-22 passages. The MB1-1 ES cells were isolated in the absence of feeder cells but in the presence of rE-HLIF these cells were cultured for 14-17 passages. The ES cells were then injected into ICR (outbred, albino) or C57BL/6J (inbred, black) blastocysts which were then transfered into pseudo-pregnant foster mothers. Both the ICR and C57BL/6J mice are homozygous for the glucose phosphate isomerase 1^b allele. Chimaeric pups were identified by coat pigmentation (only foster mothers which became pregnant were counted in estimating the number of progeny). Tissue chimaerism was estimated using glucose phosphate isomerase strain differences. The extent of tissue chimaerism was determined in two D3-ICR (numbers 1 and 2) and two D3-C57BL/6J chimaeras (numbers 3 and 4). Tissues analysed: C, coat; Bl, blood; Sp, spleen; P, pancrease; Li, liver; T, thymus; H, heart; Lu, lungs; G, gonads; K, kidneys; M, muscle; B, brain; Sa, salivary gland. Male chimaeras were mated to ICR or C57BL/6J mice and offspring identified by coat pigmentation.

calf serum (Nicola, N.A. and Metcalf, (1986) D. J.Cell Physiol. 128:160-188). Specific cell-associated ^{125}I-LIF was determined by cold competition.

Figure 3 illustrates the specific saturable and high affinity binding of ^{125}I-LIF to the ES cells EKcs-1 and the EC cells PCC3-A and F9. The number of LIF receptors per cell derived from these Scatchard plots were 295, 190 and 330, respectively, with apparent dissociation constants at 4°C of approximately 90 pM. This compares with the M1 cell line, a LIF-responsive monocytic leukaemia, which displays 50-200 LIF receptors/cell with an apparent dissociation constant of 50-150 pM. All other ES and EC cells tested - D3, NG2, PC13 and P19 - bound similar levels of LIF (data not shown).

The binding of ^{125}I-LIF to M1 cells, EKcs-1 and PCC3-A was also found to be in competition with unlabelled recombinant and native murine and human LIF, but not with the range of other hormones and factors, (including several which act on embryonic cells): insulin, IGF-I, IGF-II, acidic and basic FGF, TGFβ, TNFα, TNFβ, NGF, PDGF, EGF, IL-1, IL-4, GM-CSF, G-CSF, Multi-CSF and erythropoietin.

9. The method according to claim 8 wherein the LIF is added to the culture medium at a concentration of from 100 to 100,000 units/ml.

10. The method according to claim 9 wherein the LIF is added to the culture medium at a concentration of from 500 to 10,000 units/ml.

11. A method according to any one of the preceding claims wherein the effective time is from 1 day to 20 weeks.

12. The method according to claim 11 wherein the effective time is from 1 to 8 weeks.

13. A method for maintaining animal embryonic stem (ES) cells in vitro while retaining their pluripotential phenotype which process comprises culturing said cells in a culture medium containing an effective amount of leukaemia inhibitory factor (LIF) under conditions sufficient to maintain said cells.

14. The method according to claim 13 wherein the culture medium is free of feeder cells.

15. The method according to claim 13 or 14 wherein the animal ES cells are derived from humans, mice, birds, sheep, pigs, cattle, goats or fish.

16. The method according to claim 15 wherein the animal ES cells are derived from mice.

25. The ES cells according to claim 23 or 24 derived from human, mouse, bird, sheep, pig, cattle, goat or fish embryos.

26. The ES cells according to claim 25 derived from mouse embryos.

27. A chimaeric animal or transgenic progeny thereof generated using ES cells which have been isolated from animal embryos according to claim 1.

28. A chimaeric animal or transgenic progeny thereof generated using animal ES cells which have been maintained in vitro according to the method of claim 13.

29. The chimaeric animal or transgenic progeny thereof according to claim 27 or 28 wherein said animal is a mouse.

30. The chimaeric animal or transgenic progeny thereof according to claims 27 or 28 or 29 wherein the ES cells contain additional genetic material inserted therein.

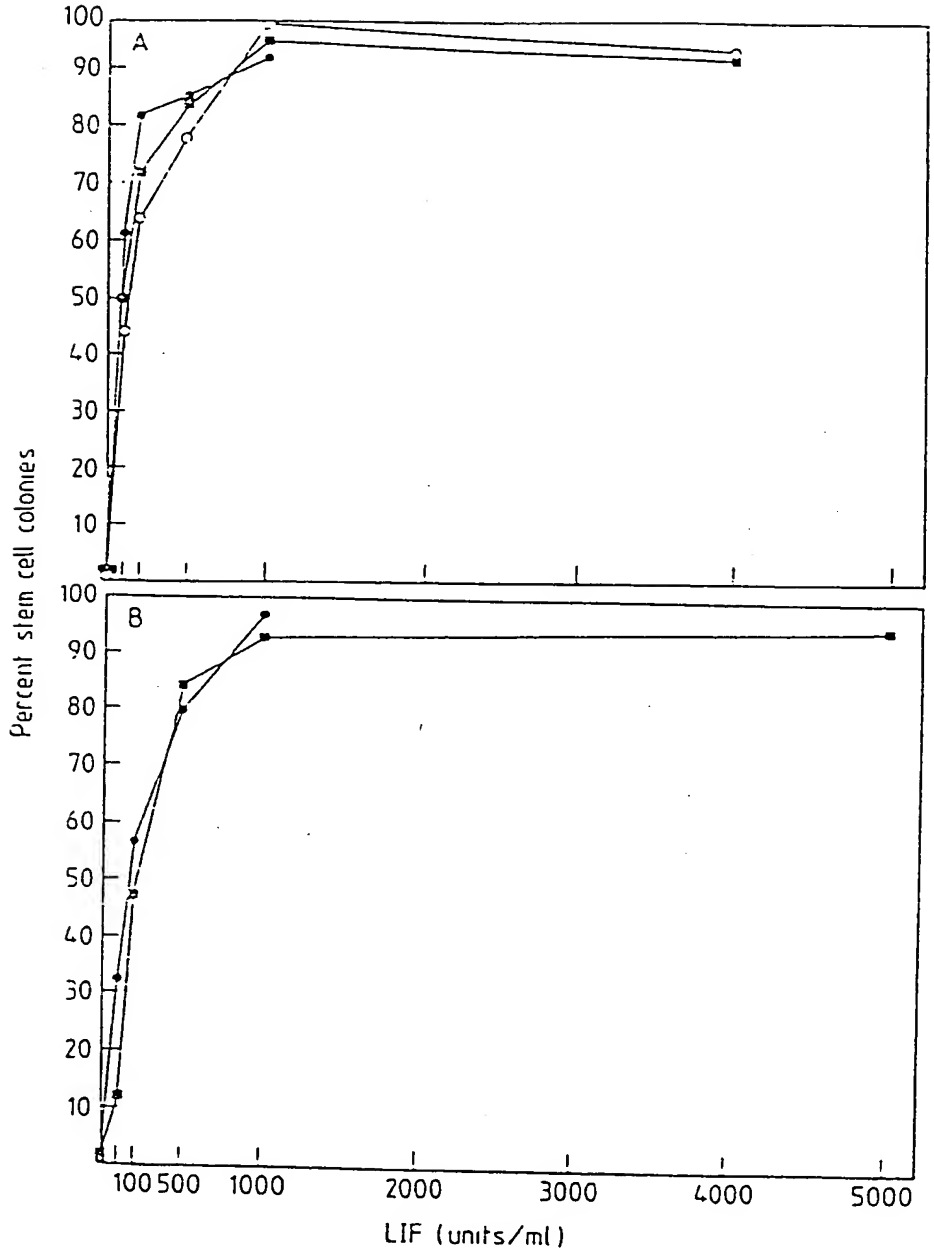

Fig.1.

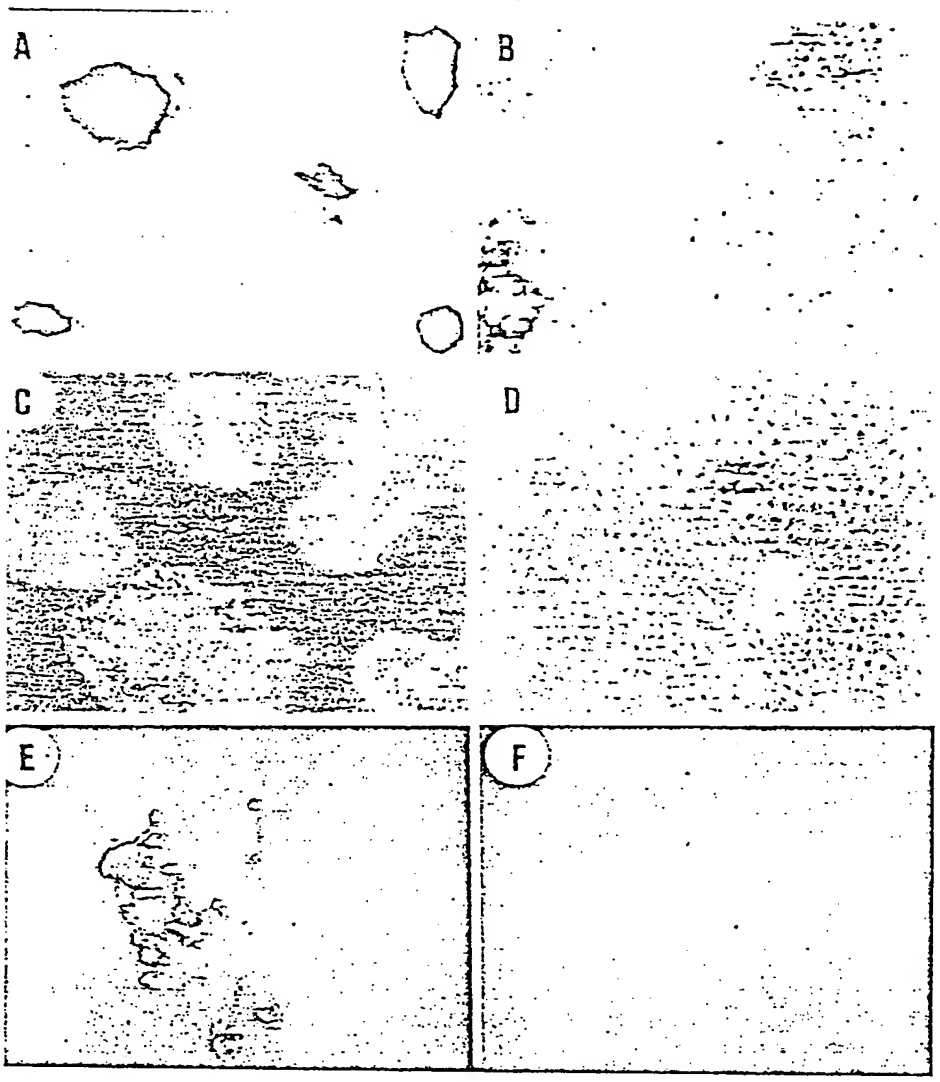

Fig.2.

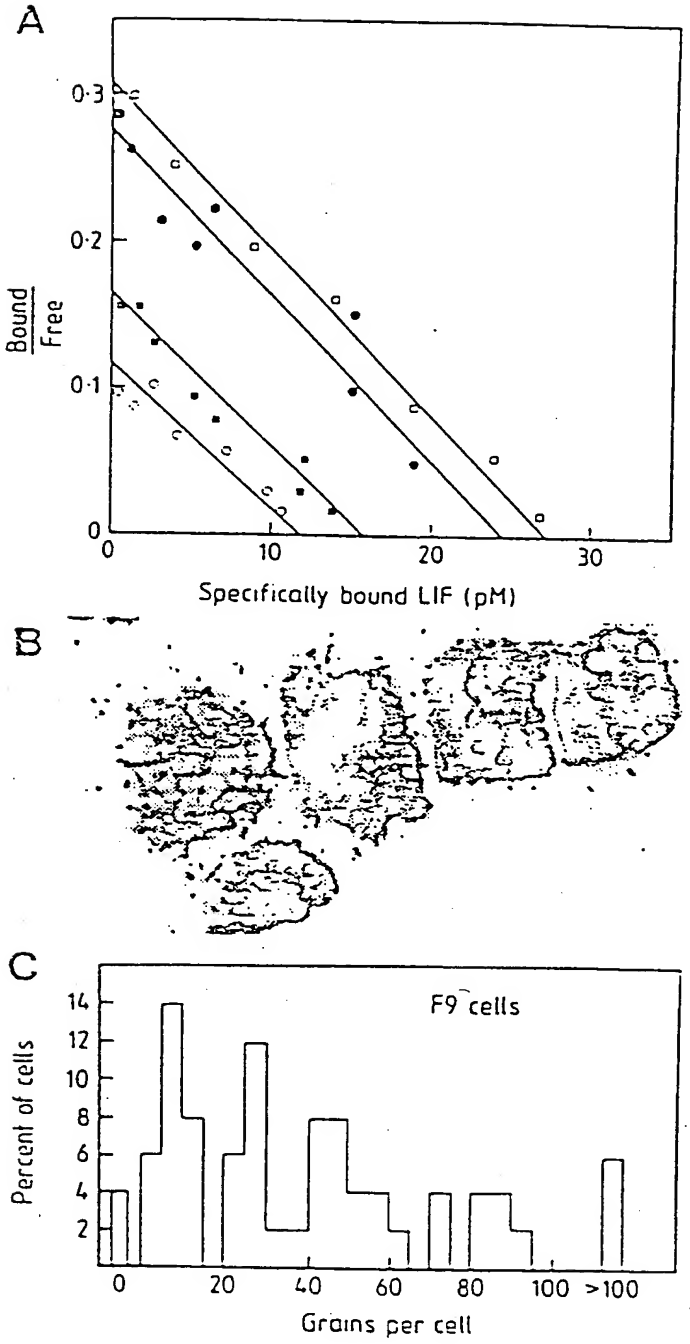
A
0·3
0·2
0·1
0
Bound / Free
0
10
20
30
Specifically bound LIF (pM)
B
C
F9 cells
14
12
10
8
6
4
2
Percent of cells
0
20
40
60
80
100
>100
Grains per cell

Fig. 3.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 89/00330

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C12N 5/00, 1/38, 15/00, A01K 67/00

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	WPI, WPIL, USPA Keywords: "Embryonic stem cells, ES cells, Leukaemia inhibitory factor, LIF"

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU: C12N 1/38, 5/00, 5/02, A61K 67/00, 67/02
Chemical Abstracts: Keywords: as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
P,X	Williams, R.L. et al. "Myeloid Leukaemia Inhibitory factor maintains the developmental potential of embryonic stem cells", Nature, Volume 336, issued 15 December 1988, (USA), see pages 684 to 687.	1 to 30
P,X,Y	Smith, A.G. et al. "Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides", Nature, Volume 336 issued 15 December 1988, (USA), see pages 688 to 690.	1 to 30
P,Y	Heath, J.K. and A.G. Smith, "Regulatory factors of embryonic stem cells", Journal of Cell Science, Supplement, Volume 10, issued 1988, (The Company of Biologists Limited 1988, Great Britain), see pages 257 to 266.	1 to 30

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2 November 1989 (02.11.89)	10 November 1989
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	J. ASHMAN

Form PCT/ISA/210 (second sheet) (January 1985)

Application Number

EP 89 90 9126

REPORT

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.5)
Y	EP-A-0 285 448 (AMRAD CORP.) * Page 21, line 28 - page 22, line 53 *	1-25	C 12 N 5/00 C 12 N 1/38 C 12 N 15/00 A 01 K 67/00
Y	PROC. NATL. ACAD. SCI. USA, vol. 85, April 1988, pages 2623-2627; N.M. GOUGH et al.: "Molecular cloning and expression of the human homologue of the murine gene encoding myeloid leukemia-inhibitory factor"	1-25	

TECHNICAL FIELDS SEARCHED (Int. Cl.5)

C 12 N

The supplementary search report has been drawn up for the claims attached hereto.

Place of search	Date of completion of the search	Examiner
THE HAGUE	13-11-1990	TURMO Y BLANCO C.E.

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

EPO FORM 1503 03.82 (P0401)

CLAIMS

1. A method for the isolation of embryonic stem (ES) cells from animal embryos in vitro which method comprises deriving and maintaining said embryos in culture medium containing an effective amount of leukaemia inhibitory factor (LIF) for a time and under conditions sufficient for the development of said ES cells.

2. A method according to claim 1 wherein the effective time is from 1 day to 20 weeks.

3. The method according to claim 2 wherein the effective time is from 1 to 8 weeks.

4. A method for maintaining animal embryonic stem (ES) cells in vitro while retaining their pluripotential phenotype which method comprises culturing said cells in a culture medium containing an effective amount of leukaemia inhibitory factor (LIF) under conditions sufficient to maintain said cells.

5. The method according to anyone of claims 1 to 4 wherein the culture medium is free of feeder cells.

6. The method according to anyone of claims 1 to 5 wherein the animal embryos are derived from humans, mice, birds, sheep, pigs, cattle, goats or fish.

7. The method according to claim 6 wherein the animal embryos are derived from mice.

8. The method according to anyone of claims 1 to 7 wherein the culture medium is Eagle's medium or

18. Embryonic stem (ES) cells derived from animal embryos *in vitro*, said cells having leukaemia inhibitory factor (LIF) associated therewith.

19. The ES cells according to anyone of claims 14 to 18 comprising additional genetic material inserted therein.

20. A cell culture preparation comprising animal Embryonic Stem (ES) cells in a culture medium comprising leukaemia inhibitory factor (LIF).

21. A chimaeric animal or transgenic progeny thereof generated using ES cells which have been isolated from animal embryos according to claim 1.

22. A chimaeric animal or transgenic progeny thereof generated using animal ES cells which have been maintained *in vitro* according to the method of claim 4.

23. The chimaeric animal or transgenic progeny thereof according to claim 21 or 22 which is a mouse.

24. The chimaeric animal or transgenic progeny thereof according to anyone of claims 21 to 23 wherein the ES cells contain additional genetic material inserted therein.

25. A method of producing a chimaeric animal comprising introducing into said animal at the preimplantation embryo stage, animal ES cells which have been isolated or maintained according to the methods of anyone of claims 1 to 13.